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Evolution of Human Immunodeficiency Virus Type 1 in Perinatally Infected Infants with Rapid and Slow Progression to Disease

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We addressed the relationship between the origin and evolution of human immunodeficiency virus type 1 (HIV-1) variants and disease outcome in perinatally infected infants by studying the V3 regions of viral variants in samples obtained from five transmitting mothers at delivery and obtained sequentially over the first year of life from their infected infants, two of whom (rapid progressors) rapidly progressed to having AIDS. Phylogenetic analyses disclosed that the V3 sequences from each mother-infant pair clustered together and were clearly distinct from those of the other pairs. Within each pair, the child's sequences formed a monophyletic group, indicating that a single variant initiated the infection in both rapid and slow progressors. Plasma HIV-1 RNA levels increased in all five infants during their first months of life and then declined within the first semester of life only in the three slow progressors. V3 variability increased over time in all infants, but no differences in the pattern of V3 evolution in terms of potential viral phenotype were observed. The numbers of synonymous and nonsynonymous substitutions varied during the first semester of life regardless of viral load, $CD4^+$-cell count, and disease progression. Conversely, during the second semester of life the rate of nonsynonymous substitutions was higher than that of synonymous substitutions in the slow progressors but not in the rapid progressors, thus suggesting a stronger host selective pressure in the former. In view of the proposal that V3 genetic evolution is driven mainly by host immune constraints, these findings suggest that while the immune response to V3 might contribute to regulating viral levels after the first semester of life, it is unlikely to play a determinant role in the initial viral decline soon after birth.

Mother-to-child transmission of human immunodeficiency virus type 1 (HIV-1) accounts for more than 95% of the cases of pediatric AIDS. Moreover, about one-third of these infected infants will develop severe symptoms of disease and/or severe immunodepression by 1 year of age. The timing of transmission, the pathogenic potential of the transmitted variants, and the host's capability to control the growth of the viral population have each been postulated to explain the observed differences in progression to disease (for a review, see reference 48). Although the precise timing of vertical transmission cannot be pinpointed, it has been proposed that detection or lack of detection of the virus in the child at birth might reflect viral transmission in utero or during the intrapartum period (18). By using highly sensitive PCR-based methods to directly detect HIV-1, we and others (3, 15, 19, 30) demonstrated that a consistent proportion of infants who are subsequently recognized as infected do not have detectable virus levels in their peripheral blood cells at birth. Further studies on the dynamics of HIV-1 replication after birth (16) and the appearance of the child's autochthonous antibodies (14, 49) indicate that HIV-1 transmission occurs mainly late in pregnancy or at delivery. Most of the investigations that have evaluated the genetic diversity of the virus found a highly homogeneous viral population in newborns, thus indicating that a limited number of variants or even one variant initiated the infection in the infants (1, 38, 54, 58, 67). However, few studies have addressed the relationship between acquired HIV-1 variants and their evolution over time in relation to disease outcome (26, 58).

An accumulating body of evidence indicates that disease progression in adults (37, 47) as well as in infants (16, 18) is directly related to the level of virus replication; multiple viral factors, such as tropism and replicative capacity, and host factors, including cellular and humoral immune responses, are likely to determine virus levels.

Genetic analysis of the third variable region (V3) of the *env* gene has provided information about the potential phenotype of the virus and host-dependent constraints, since this region contains determinants involved in a number of biological properties of the virus, including tropism and cytopathicity (5, 8, 11, 27, 55), as well as recognition sites for both humoral and cellular T-cell immune responses (45, 50–52, 59, 60). Indeed, specific amino acid changes in V3 have been associated with the appearance of syncytium-inducing (SI) viral variants (10, 24) and resistance to neutralizing antibodies (63), and it has been proposed that a rapid evolution of antigenic sites such as the V3 region might exceed the capacity of the immune system to control virus growth (41, 42). According to this theory, the V3 region would evolve more rapidly in individuals progressing to AIDS than in those who remain asymptomatic longer (41, 42). A study of twins with different disease courses reported a higher increase in sequence diversity, paralleled by a lower immune response, in the infant who progressed rapidly to AIDS (rapid progressor) than in the slow-progressor infant (26). Higher rates of sequence divergence and nonsynonymous nucleotide substitutions within the V3 region have also been

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reported for two children who progressed faster to AIDS compared to four slower-progressing infants (58). However, it has recently been demonstrated that an increase in genetic diversity was correlated with a slower $CD4^+$-cell decline and a prolonged asymptomatic period in adults who had been monitored since primary infection (64). Furthermore, the observation that the rate of nonsynonymous nucleotide substitutions was higher in slow progressors than in subjects who progressed rapidly to AIDS and was correlated with the duration of the immunocompetent period (33, 64) leads to the proposal that the evolution of the viral population is driven mainly by host immune system selective forces.

To better clarify the transmitted variants and their evolution in relationship to disease outcome, we studied the V3 regions of the viral variants present in samples collected at delivery from five mothers and collected sequentially over the first year of life from their infected infants. The evolution of the V3 region was compared to the $CD4^+$-cell number, plasma HIV-1 RNA levels, biological phenotype of the virus, and timing of seroconversion.

MATERIALS AND METHODS

Patients. Five HIV-1-infected mother-infant pairs were examined in this study. The women attended the Gynecology and Obstetrics Department of the University of Padova. Maternal samples were collected within 7 days of delivery. The clinical stage according to the Centers for Disease Control (CDC) (6) and the $CD4^+$-cell count were provided with the samples. Peripheral blood samples from the children were supplied by the Pediatrics Department of the University of Padova. All of the children were full-term infants born by spontaneous vaginal delivery; none were breast fed. The infection status of the children was defined by virus isolation and PCR as previously described (13, 15). The children were followed clinically and immunologically every month during the first 3 months of life and then every 2 to 3 months; clinical and immunological statuses were defined according to the CDC criteria (7).

Sample preparation. Heparinized peripheral blood samples were centrifuged over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradients; plasma was recovered from the upper phase, centrifuged at $1,000 \times g$ for 15 min to ensure cell-free specimens, and stored at −80°C until further analysis. Peripheral blood mononuclear cells (PBMCs) were recovered from the top of the Ficoll gradient, washed twice with phosphate-buffered saline, and counted. Two million PBMCs were lysed for 1 h at 56°C in 500 μl of TE buffer (10 mM Tris-HCl [pH 8] and 0.1 mM EDTA) containing 0.001% Triton X-100, 0.0001% sodium dodecyl sulfate, and 600 μg of proteinase K/ml. After lysis, proteinase K was inactivated by heating the mixture for 15 min at 94°C. Detection of HIV-1 by PCR was performed directly on aliquots of the lysed cells as previously reported (15). The remaining cells were resuspended in RPMI medium supplemented with 10% fetal calf serum and 10% dimethyl sulfoxide and then were cryopreserved for biological assays.

Nested PCR and cloning. Nested PCR amplifications of proviral DNA were carried out on lysed PBMCs. Only one sample was processed at a time to avoid cross-contamination. The V3 region was amplified with the outer primer pair V3A (5′ TACAATGTACACATGGAATT 3′) and V3D (5′ ATTACAGTAGA AAAATTCCCC 3′) and the inner primers V3B (5′ TGGCAGTCTAGCAGA AGAAG 3′) and V3C (5′ CTGGGTCCCCTCCTGAGG 3′). The primer-binding sites were highly conserved between sequences of geographical variants of HIV-1 (39); their positions in $HIV\text{-}1_{IIIB}$ (clone HXB2) are as follows (5′ to 3′): V3A, nucleotides (nt) 6957 to 6976; V3B, nt 7009 to 7028; V3C, nt 7314 to 7331; and V3D, nt 7361 to 7381 (57). The first-round PCRs were performed with 25 μl of lysed PBMCs, corresponding to 10^5 cells, in a 100-μl reaction mixture containing 20 nmol of each deoxynucleoside triphosphate, 50 pmol of each of the outer primers, and 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Forty cycles were carried out in a Gene Amp PCR System 9600 thermal cycler (Perkin-Elmer), each consisting of 30 s at 94°C, 30 s at 49°C, and 30 s at 72°C, followed by one cycle at 72°C for 5 min. Five microliters of the first-round PCR was amplified in a nested PCR with 80 pmol of the inner primers; 40 cycles were run, each consisting of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, followed by a final extension at 72°C for 5 min. Appropriate negative controls (10^5 HIV-1-negative A301 cells, lysis buffer, and distilled water) were included in each set of reactions. All negative controls from the first round of amplification were included in the second amplification step. The sensitivity of nested PCR was assessed by using 8E51 cells, which contain a single proviral DNA copy of HIV-1 per cell, serially diluted in 10^5 PBMCs separated from heparinized blood of a healthy donor; 5 of 10 samples that were calculated to contain one 8E51 cell yielded the expected product following nested PCR. This result is in agreement with the reported sensitivity of nested PCR to amplify a single HIV-1 copy (36).

Two separate PCRs for each sample were performed for subsequent cloning. The amplified products were separated on a 2.5% preparative low-melting-point agarose gel (NuSieve; FMC, Rockland, Maine). The band of the appropriate size (322 bp) was excised and purified by using the Wizard PCR Preps DNA Purification System (Promega, Madison, Wis.) according to the manufacturer's instructions and then cloned by using the TA cloning kit (Invitrogen, San Diego, Calif.). Four nanograms of the amplified products was ligated to 50 ng of pCRII vector with 4 U of T4 ligase for 16 h at 14°C and transformed into competent cells of *Escherichia coli* INVαF′. Clones were color selected on indicator plates containing 50 μg of ampicillin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. White colonies were selected and amplified in culture, and bacterial DNA was recovered by using a DNA purification kit (BIO101, RPM Inc., Vista, Calif.), according to the manufacturer's instructions. The presence of the insert in the plasmid clones was screened by digestion with 10 U of *Eco*RI restriction enzyme (Boehringer, Mannheim, Germany); the insert was screened for appropriate size and relative quantity by electrophoresis on an 0.8% agarose gel.

Sequencing. Nucleotide sequencing of the cloned PCR products (9 to 24 clones for each sample) was performed by the Sanger dideoxynucleotide method, using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio). Each clone was sequenced in the forward and reverse directions with the V3B and V3C primers. One microgram of the double-stranded plasmid DNA was mixed with 12 pmol of primer and heat denatured for 5 min; the annealing step was carried out for 1 min at room temperature. The labeling reaction was performed on ice for 3 min in a final volume of 20 μl containing 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 25 mM NaCl, 5 mM dithiothreitol, 75 nM each deoxynucleoside triphosphate, 5 μCi of [α-^{33}P]dATP (Amersham, Buckinghamshire, United Kingdom), and 3 U of Sequenase. Four-microliter aliquots of the mixtures were combined with 2.5 μl of each termination mixture (ddATP, ddCTP, ddTTP, and ddGTP). After 5 min at 37°C, 4 μl of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) was added. The sequencing products were denatured at 80°C for 3 min, and 10-μl aliquots were resolved by electrophoresis on 8% polyacrylamide denaturing gels in a sequencing apparatus (GIBCO-Bethesda Research Laboratories, Gaithersburg, Md.); the gels were then exposed for 16 h to X-OMAT AR film (Eastman Kodak, Rochester, N.Y.).

To calculate the rate of misincorporations introduced during PCR, cloning, and sequencing, 10 clones from two different PCR amplifications of 8E51 cells were sequenced as described above; only one point mutation was observed, corresponding to a misincorporation rate of 1/1,890 (0.05%).

Sequence analysis. The sequence analysis program Microgenie (IntelliGenetics, Inc., Mountain View, Calif.) was used to record and translate V3 sequences. Nucleotide and translated amino acid sequences from each mother-child pair were aligned by using the Clustal V program (25). The alignments were then adjusted by hand, and nucleotide gaps were introduced to maintain translation integrity. Consensus amino acid sequences were derived by assigning the deduced amino acid found in more than 50% of the clones to each position.

Phylogenetic analysis was conducted with programs from version 3.52c of the Phylogeny Inference Package (PHYLIP) (21). Nucleotide sequence distances for all pairwise sequence comparisons were estimated by means of the generalized two-parameter (maximum-likelihood) model, which uses the transition probability formulas of Kishino and Hasegawa (29) (program DNADIST). Intrasample and intersample sequence variations were expressed as the mean distance for all pairwise comparisons between sequences obtained within a sample and from different samples, respectively. Phylogenies were reconstructed for each mother-child pair by both the neighbor-joining method (53) (program NEIGHBOR) and the Fitch-Margoliash distance method (23) (program FITCH); both tree construction methods were employed in order to increase confidence in the reconstructed phylogenies. Bootstrap resampling (20) was applied to the neighbor-joining trees (programs SEQBOOT and CONSENSE) to assign approximate confidence limits to individual branches.

The genetic variability for each sample was calculated from the amino acid sequences by using the Simpson index, calculated as $D = \Sigma_i n_i^2/n^2$, where n_i denotes the number of type i sequences in the sample and n denotes the total number of sequences (41). The number of synonymous substitutions per potential synonymous site (D_s) and the number of nonsynonymous substitutions per potential nonsynonymous site (D_n) for each set of sequences from the different time points were calculated by using the method of Nei and Gojobory (40), incorporating the Jukes-Cantor correction for multiple substitutions, as implemented in the MEGA program (32).

Viral DNA and RNA quantitation. HIV-1 proviral DNA in cells and genomic HIV-1 RNA in plasma samples were quantified by using competitive DNA-PCR and competitive reverse transcription-PCR, respectively, as previously reported (16). Briefly, to quantify proviral DNA, replicate portions of lysed cells (15 μl of cell sample, corresponding to 60,000 cells) were amplified along with 2 μl of increasing copy numbers of competitor plasmid pSPLI-II (16). To quantify RNA, virion-associated RNA was obtained from plasma by an affinity capture method; replicate portions of RNA samples (2 μl, corresponding to 30 μl of plasma) were reverse transcribed along with 2 μl of increasing copy numbers of competitor RNA transcribed from pSPLI-II. Amplification was carried out in a 100-μl final volume with 100 pg of the primer pair 1-2II, specific for highly conserved regions of HIV-1 and corresponding to the following HIV-1 MN sequences (5′-3′): nt 696 to 723, upstream of the first 5′ splice site (primer 1), and nt 914 to 889 within

gag (primer 2II). To increase the sensitivity of the assay, the sense primer was radiolabeled with [γ-^{32}P]dATP. Forty-five amplification cycles were run, each consisting of 50 s at 94°C, 45 s at 62°C, and 50 s at 72°C. A 30-μl aliquot of each 100-μl PCR mixture was run on a polyacrylamide gel and exposed to an X-ray film for 2 to 4 h at −80°C. The sizes of the competitor and wild-type amplified products were 240 and 218 bp, respectively. The peak areas of the amplified bands were measured by densitometric scanning (ULTRASCAN XL; LKB, Pharmacia). The logarithm of the ratio between the optical density values of competitor and wild-type amplified products (*y* axis) was plotted against the logarithm of the competitor copy number (*x* axis), and a linear regression curve was extrapolated.

Viral phenotype analysis. Viral isolation was performed as previously reported by culturing patient PBMCs with phytohemagglutinin-stimulated donor PBMCs (13). Isolates were defined as rapid/high, slow/high, or slow/low according to the day of p24 antigen appearance and the levels of p24 in the supernatants of the primary coculture (13). HIV-1 isolates obtained from the primary cocultures were propagated by a single short-term passage (7 days) in PBMCs; supernatants were collected, centrifuged at 15,000 × *g* for 1 h, and then filtered through 0.22-μm-pore-size filters (Millipore, Bedford, Mass.). The virus content was quantified by measuring HIV-1 p24 protein. Individual isolates were aliquoted, stored at −80°C, and then used to evaluate infectivity in primary monocyte-derived macrophages and peripheral blood lymphocytes and in the T-cell line MT-2, as previously described (43).

Autochthonous-antibody production. De novo synthesis of antibodies to HIV-1 in infants was estimated by Western blot profiles and/or an enzyme-linked immunosorbent assay with HIV-1-derived synthetic peptides as antigens, exactly as previously reported (14, 17).

Nucleotide sequence accession numbers. The sequences reported here have been deposited in the GenBank database under accession no. U74767 to 74976 and U74987 to U75185.

RESULTS

Clinical status of patients. The characteristics of the five mother-child pairs studied are summarized in Table 1. Maternal blood samples were collected at delivery in three cases (M2, M3, and M4) and at 7 days after delivery in two others (M1 and M5). All of the mothers were of Italian origin and formerly were intravenous-drug users. All mothers but one were asymptomatic during their pregnancy, and none underwent antiretroviral therapy during pregnancy.

Four of the infants born to these mothers were tested for HIV-1 infection by PCR and virus culture at birth; infant C5 was tested on day 7 and was found to be HIV-1 positive at this first examination. The other infants were found to be HIV-1 negative at birth but were HIV-1 positive at their second examination, which was performed within 1 month of birth (13, 16). According to the CDC criteria (7), the children were classified as asymptomatic (category N), mildly symptomatic (category A), moderately symptomatic (category B), or severely symptomatic (category C); the immunological status was defined as class 1 (no immunodepression), 2 (moderate immunodepression), or 3 (severe immunodepression) on the basis of the number of $CD4^+$ cells per microliter as adjusted for age (7). Infants C4 and C5, who developed the symptoms listed in category C and/or severe immunodepression within the first year of life, were classified as rapid progressors, according to the definition of the Consensus Workshop on Pediatric AIDS (61). Infant C4 developed *Pneumocystis carinii* pneumonia by 3 months of age, while infant C5 showed severe immunodepression (<750 $CD4^+$ cells/μl) by 5 months of age and developed encephalopathy by 10 months of age; infants C4 and C5 started therapy with zidovudine at 4 and 6 months, respectively. The remaining three infants (C1, C2, and C3) were classified as slow progressors; within the first year of life, they showed lymphadenopathy (C1 and C3) or hepatosplenomegaly (C2), and over the course of 4.5 years (mean follow-up time), none has developed severe immunodepression or progressed to category C. Sequential samples collected over the first year of life at the time points listed in Table 1 were employed for genetic analysis.

TABLE 1. Times of sampling and clinical data for mother-child pairs

Pair	Sample	Code	Clinical stage[a]	HIV-1 detection at birth	Time of testing for genetic analyses (days after delivery)	HIV-1 DNA copies/10^5 PBMCs
1	M1	2222	IV		7	382
	C1.1	2243	N1	−	37	296
	C1.2	2415	A1		184	469
	C1.3	2564	A1		296	343
2	M2	891	II		1	73
	C2.1	982	N1	−	77	327
	C2.2	1143	A1		186	98
	C2.3	1525	A1		400	265
3	M3	892	II		1	92
	C3.1	996	A1	−	85	314
	C3.2	1145	A1		190	112
	C3.3	1461	A1		384	335
4	M4	1101	II		1	237
	C4.1	1102	B1	−	29	591
	C4.2	1223	C1		90	480
	C4.3	1588	C2		315	2,237
5	M5	2693	II		7	193
	C5.1	2663	A1	+	7	26
	C5.2	2726	B1		48	1,386
	C5.3	2848	B3		137	1,096
	C5.4	3223	C3		390	1,016

[a] The mother's clinical stage was defined according to the CDC criteria for adults (6). The child's clinical stage was defined according to the CDC criteria for children less than 13 years of age (7).

Nucleotide sequence variability. To examine the origin and evolution of HIV-1 genotypes in infected infants, proviral sequences from the maternal PBMC sample at delivery and the infants' sequential samples were amplified by nested PCR and cloned into the vector pCRII; a panel of 9 to 24 clones, each containing sequences spanning the V3 domain and flanked 5′ and 3′ by 60 and 21 bp, respectively, was sequenced from each of the studied samples.

The infants' samples showed a more homogeneous viral population (0 to 1.53% intrasample mean nucleotide distances) than the maternal samples (1.34 to 4.12% intrasample mean nucleotide distances) (Fig. 1A). The initial genetic variations of sequences from infants C1, C2, C3, C4, and C5 were 0.22, 0.37, 1.20, 0.71, and 0%, respectively. Heterogeneity increased with age in all of the infants with the exception of C3; however, after an initial decrease (from 1.20 to 0.81%), an increase in sequence variability (from 0.81 to 1.53%) was detected in this child as well. The increase in HIV-1 variability over time was confirmed by an intersample analysis between sequences from the first sample and each subsequent sample, which showed an increase in the mean nucleotide distance values (Fig. 1A).

The mean nucleotide distances between sequences from the

A

PAIR 1

	n	M1	C1.1	C1.2	C1.3
M1	9	1.34			
C1.1	20	1.76	0.22		
C1.2	14	1.77	0.22	0.23	
C1.3	14	2.41	0.85	0.86	0.72

PAIR 2

	n	M2	C2.1	C2.2	C2.3
M2	18	2.02			
C2.1	17	4.10	0.37		
C2.2	22	4.27	0.48	0.56	
C2.3	19	4.51	0.97	1.03	1.38

PAIR 3

	n	M3	C3.1	C3.2	C3.3
M3	22	1.62			
C3.1	19	3.83	1.20		
C3.2	24	3.27	1.10	0.81	
C3.3	22	3.11	1.77	1.37	1.53

PAIR 4

	n	M4	C4.1	C4.2	C4.3
M4	22	4.12			
C4.1	21	4.20	0.71		
C4.2	20	4.44	0.89	0.91	
C4.3	21	4.51	1.19	1.32	1.39

PAIR 5

	n	M5	C5.1	C5.2	C5.3	C5.4
M5	20	1.62				
C5.1	19	0.95	0.00			
C5.2	22	0.98	0.02	0.05		
C5.3	22	1.04	0.10	0.12	0.20	
C5.4	21	1.54	0.60	0.62	0.69	1.15

B

	n	PAIR 1	PAIR 2	PAIR 3	PAIR 4	PAIR 5
PAIR 1	57	0.93				
PAIR 2	77	9.65	2.14			
PAIR 3	87	9.71	10.42	2.13		
PAIR 4	84	8.85	13.35	10.87	2.58	
PAIR 5	104	12.62	13.27	12.21	14.05	0.65

FIG. 1. Nucleotide sequence variation in the V3 region of the *env* gene in five mother-infant pairs, showing intrasample and intersample mean nucleotide distances within mother-infant pairs (A) and between mother-infants pairs (B). Shown on the diagonal are the mean nucleotide distances from all pairwise intrasample (A) and intrapair (B) comparisons. Shown below the diagonal are the mean distances between intersample (A) and interpair (B) comparisons. n, number of sequences analyzed for each sample (A) and pair (B).

mother and those in the child's first sample were 1.76, 4.10, 3.83, 4.20, and 0.95% for pairs 1, 2, 3, 4, and 5, respectively (Fig. 1A), while the lowest nucleotide distances between sequences from mother's and child's first sample were 0.54, 2.79, 2.21, 0, and 0% for pairs 1, 2, 3, 4, and 5, respectively (not shown); interestingly, these values correlated with the child's age at time of first sampling (Table 1). The divergence between the sequences of the child and the mother increased over time, with the exception of child C3, who showed the highest mean nucleotide variability (1.77%) over time (Fig. 1A).

Analysis of all sequences within and between pairs disclosed that mean nucleotide divergence values within each mother-child pair fell within the range of intrasample variability (from 0.65 to 2.58%); this was significantly lower ($P = 0.0026$ [Mann-Whitney test]) than the interpair variability (8.85 to 14.05%) (Fig. 1B).

Phylogenetic analyses. A phylogenetic analysis of the sequences of the five mother-child pairs with reference sequences from the five *env* clades of HIV-1 disclosed that all of the sequences belonged to clade B (data not shown), in agreement with the Italian origin of the patients. A phylogenetic tree was reconstructed by using the neighbor-joining method for all 409 sequences obtained for the mothers and infants in this study (Fig. 2). The results showed that the sequences from each mother-infant pair clustered together; the five pairs were clearly discriminated and well confined within subtrees. High bootstrap values were observed for the five mother-infant sequence sets, as would be expected for epidemiologically unrelated individuals, and excluded the possibility of contamination of the PCRs. Within the phylogenetic tree, the infants' sequences extended further from the putative ancestral node than those of the corresponding mothers, thus indicating a

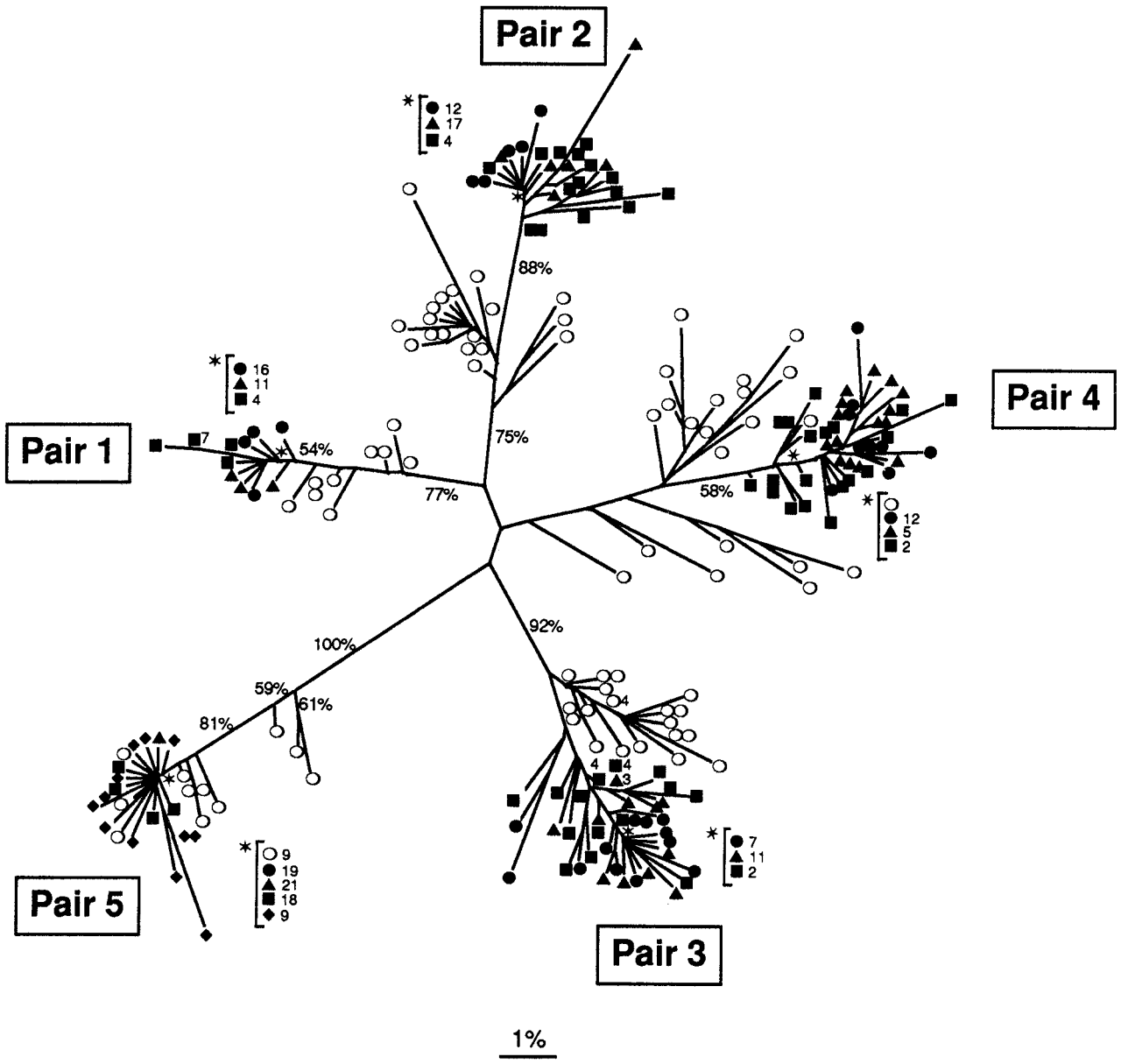

FIG. 2. Unrooted neighbor-joining tree of 409 V3 nucleotide sequences obtained from the five mother-child pairs. Branch lengths are drawn to scale. The scale bar corresponds to 1% nucleotide sequence divergence. Bootstrap values are expressed as percentages for each branch and represent the percent occurrence of that branch per 100 bootstrap replicates. Open symbols denote maternal sequences; closed symbols indicate children's sequences. ●, first sample; ▲, second sample; ■, third sample; ◆, fourth sample. Numbers refer to the number of identical sequences identified in a given sample. *, position in the tree of the set of sequences included in the bracket.

more divergent evolution. Within the subtrees, moreover, the maternal sequences were distributed into different branches, thus suggesting a pattern of multiple distinct lineages; conversely, in each infant's sequence set, most of the sequences from the first time point were clustered in a single branch, thus suggesting a monophyletic origin of the infection.

To further investigate the relationship between the maternal and infant sequences, phylogenetic analysis was performed separately for each mother-child nucleotide sequence set (Fig. 3). In all pairs, the infants' sequences from the first sample were more homogeneous than those of the corresponding mother and clustered tightly within a single branch in the phylogenetic tree, forming a monophyletic group. In pairs 2 and 3, viral variants of the child clustered distinctly from sequences of the mother; this distinction was supported by relatively high bootstrap values (79% for pair 2 and 73% for pair 3). In pairs 4 and 5, some maternal and infant sequences were intermingled: in pair 4, two minor maternal viral variants that were separated from the main group clustered with the child's sequence group, and in pair 5, the predominant maternal sequence was identical to the major sequence detected in the child at birth. In pair 1, maternal and infant sequences were distinct at the nucleotide level, but the maternal sequence displayed by two of nine clones, and located in the phylogenetic tree nearest to the child's sequences, was identical at the amino acid level to the predominant viral variant detected in the first sample from the child (Fig. 4). In infants C3 and C4, the sequences that showed the most divergence from those of

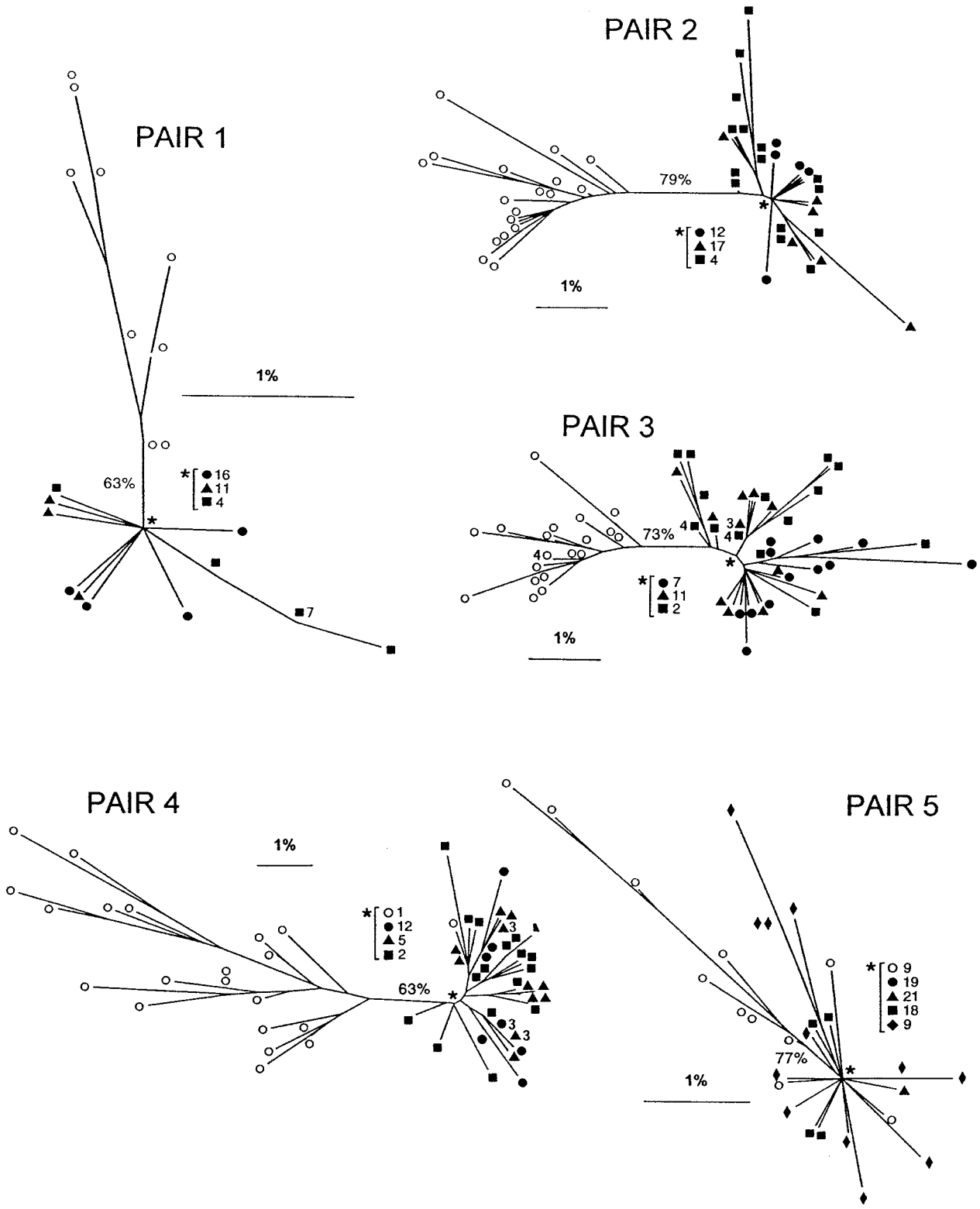

FIG. 3. Neighbor-joining trees for each of the five mother-infant pairs. Branch lengths are drawn to scale. The scale bars correspond to 1% nucleotide sequence divergence. The number at the node indicates the proportion of support in 2,000 bootstrap replicates. Open symbols indicate the mother's sequences; closed symbols indicate the child's sequences. ●, first sample, ▲, second sample, ■, third sample, ◆, fourth sample. Numbers refer to the number of identical sequences identified in a given sample. *, position in the tree of the set of sequences included in the bracket.

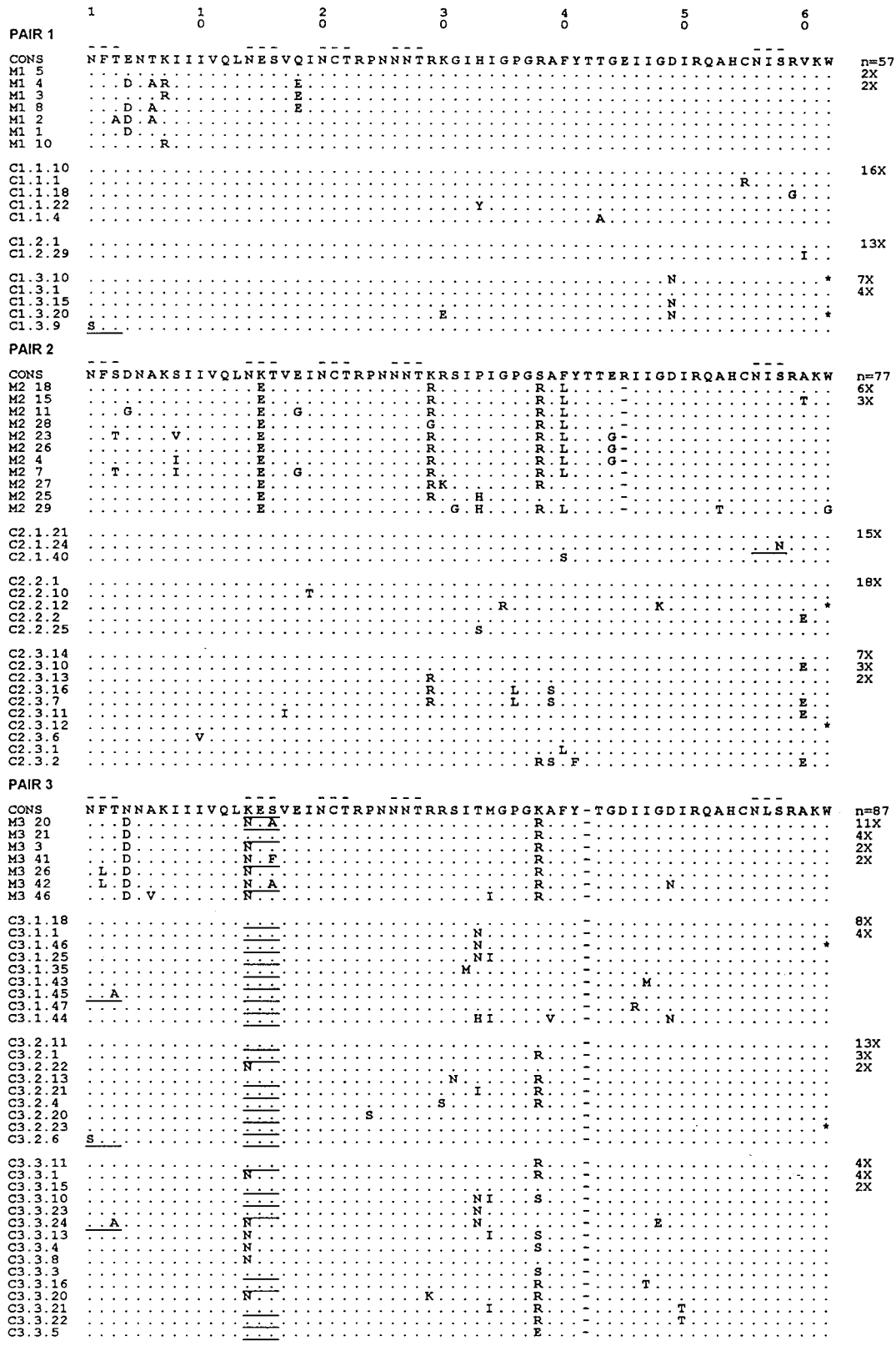

FIG. 4. Multiple alignment of deduced amino acid sequences of the V3 regions of 409 sequences derived from the five mother-infant pairs. Position 1 corresponds to amino acid 276 of the HXB2 envelope protein. Sequences are aligned against the consensus (CONS) sequences derived for each mother-infant pair. The number of clones obtained from each pair is shown at the end of the consensus sequence. The frequency of clones with identical amino acid sequences for each sample is given at the end of the sequence. Dots indicate identity with the reference sequence, dashes represent gaps introduced to maximize alignment, and asterisks represent stop codons. Potential N-linked glycosylation sites are indicated by three dashes above the alignment. The loss of a potential N-linked glycosylation site is indicated by underlined letters.

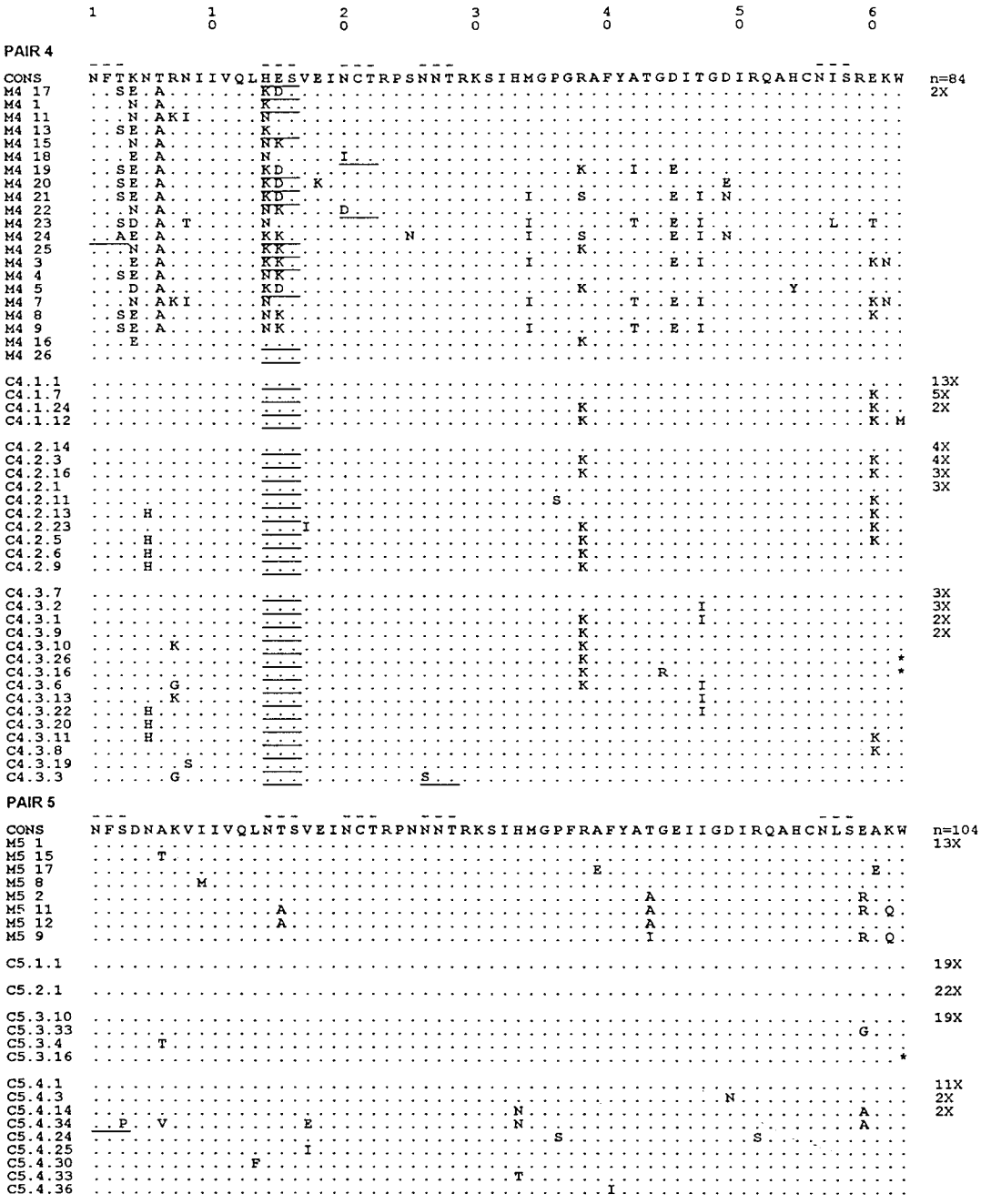

FIG. 4—*Continued.*

the corresponding mothers were detected at the first time point (Fig. 2 and 3); in both cases, these sequences corresponded to variants with substitutions seen only once in the sequence set (clones C3.1.44 and C3.1.47 in child C3 and clone C4.1.12 in child C4 [Fig. 4]). These sequence changes could represent either in vitro-introduced misincorporations or randomly occurring substitutions acquired during the first cycles of viral replication soon after birth.

The evolutionary pattern of the viral variants in C1, C2, C3, and C4 resembled a "star" phylogeny with branches radiating from the point at which the majority of the sequences detected at the first time point were clustered. In infant C3, some of the sequences derived from the latest sample were located within the phylogenetic tree nearest to the maternal sequence (Fig. 3). From this analysis, we could not discriminate between a phenomenon of convergent evolution or the emergence of additional transmitted maternal variants later in the child's life.

Amino acid sequence analysis. The deduced amino acid sequences of the V3 loop and flanking regions obtained for the five mother-child pairs are presented in Fig. 4. The coding potential of the envelope open reading frame was maintained in most of the sequences, with 15 inactivating mutant stop

codons (nonsynonymous substitutions TGG→TAG or TGA at position 62) detected in 75,573 bases sequenced; this substitution was observed at the same position in a previous study (1). No frameshifts were observed among the 409 sequences, and the two cysteines involved in disulfide bridge formation of the V3 loop (at positions 21 and 55 [Fig. 4]) were conserved in all but one sequence (i.e., cysteine to arginine in clone C1.1.1).

While the maternal virus population displayed a swarm of genetically distinct variants, in the first sample from each infant there was a predominant viral variant, representing from 42% (C3) to 100% (C5) of the viral population. A few minor viral variants were also detected; the differences between major and minor intrasample variants were due to randomly occurring substitutions, which were observed primarily in only one or a few sequences within each set of clones. A comparison of the child's amino acid sequences with those of the corresponding mother revealed that in all of the infants the predominant variant was the one most closely related to the maternal sequence(s). In particular, in pairs 1 and 4, the child's predominant variant was identical to a minor maternal variant, while in pair 5, the child's predominant variant was identical to a major maternal variant. For mothers M4 and M5, these variants corresponded to those intermingled with the infants' sequences in the phylogenetic tree; for mother M1, this variant corresponded to the one (M1.5) showing the smallest nucleotide distance (0.54%) from the child's predominant sequence (C1.1.10). In infants C2 and C3, none of the sequences were found to be identical to those of the corresponding mother at either the nucleotide or the amino acid level; nevertheless, within each clone set, the major variant showed the smallest nucleotide and amino acid divergence from the maternal sequences. It is noteworthy that a length polymorphism was observed in pair 2, with all of the child's sequences showing an amino acid insertion at position 45. Interestingly, specific amino acid residues, i.e., serine at position 38 and phenylalanine at position 40, were detected in most of the child's sequences, but only in a minor maternal genotype (M2.25). In pair 3, a specific amino acid deletion at position 42 was observed in both the mother's and child's sequences; although no specific maternal variant more closely related to the child's sequences could be identified, the predominant variant in the child displayed the highest degree of homology with the maternal sequences. Therefore, it is likely that the predominant variant in the first sample constituted the form that initiated the infection in the child and that minor variants were generated from the major one by point mutations.

Analysis of sequences derived from subsequent samples disclosed that the predominant variant persisted over time in each infant, even though it became less represented; indeed, its percentage within the total viral population from the first to the latest sample changed from 80 to 28%, 88 to 37%, 42 to 9%, 62 to 14%, and 100 to 52% in infants C1, C2, C3, C4, and C5, respectively. In addition to the expansion of minor variants, new variants were also detected in the subsequent samples. Based on a comparison of the phylogenetic and amino acid analyses, with the exception of those of child C3, none of these variants appeared more closely related to the maternal variant than the predominant variant detected in the first sample. Both conservation and divergence were observed in the central motif of the V3 loop within the mother-infant sequence sets. In pair 1, all sequences of both mother and child retained the GPGR motif; the GPGS motif was highly conserved in child C2, while all of the sequences of mother M2 except one (M2.25) showed the GPGR motif. Both the GPGK and GPGR motifs were detected in the maternal and child sequences of pair 4, while most of the sequences from pair 5 displayed the GPFR motif. In the sequence set derived from the samples of child C3, a shift in the central GPGK motif (detected in 100% of clones from the first sample) to GPGR (detected in 100% of maternal clones) was observed in 25 and 54% of clones obtained from the second and third samples, respectively.

The addition of carbohydrate chains to potential glycosylation sites may modulate the host immune response by obscuring linear epitopes, thus facilitating escape variants (2, 28, 56). Five potential N-linked glycosylation sites (NXT or NXS sequons), located at positions 1, 14, 20, 26, and 56, are present in the V3 and flanking regions (Fig. 4). Four sites (located at positions 1, 20, 26, and 56) were fairly conserved among the clonal sequences of all five mother-child pairs. One site, located at position 14, was conserved in all of the sequences of pairs 1, 2, and 5. In pair 4, this site was absent in all of the child's sequences and in 13 of 22 maternal sequences. In pair 3, this site was absent in the majority of the maternal sequences and in all of the child's sequences from the first sample; in subsequent samples, however, this glycosylation site was detected in an increasing number of sequences (2 of 24 and 9 of 22 in the second and third samples, respectively). Given that the GPGR and GPGK central motifs are common in the strains of clade B and that the potential N-linked glycosylation sites are quite variable among primary HIV-1 isolates (39), the features noted in the subsequent samples of child C3 may support a phenomenon of convergent evolution rather than a transmission of multiple maternal genotypes to the infant.

V3 evolution in rapid- and slow-progressor infants. Figure 5 reports the intrasample variability and the numbers of synonymous and nonsynonymous nucleotide substitutions calculated for the V3 sequences from each child's sample, along with the sequential plasma HIV-1 RNA values and $CD4^+$-cell counts (16). As shown in Fig. 5A, all infants exhibited a rapid increase in plasma viral RNA during the first 4 to 6 weeks of life. After this period, viral RNA levels decreased by at least 10-fold in slow-progressor infants C1, C2, and C3 but remained at high levels (>1,000,000 RNA copies/ml of plasma) in rapid-progressor infants C4 and C5. Viral decline did not appear to correlate with the onset of an HIV-1 humoral immune response, as autochthonous antibodies could be detected in four of the five infants, including the two rapid progressors. The time of seroconversion, estimated as the mean period between the child's age at the last negative finding and the first positive finding for autochthonous-antibody production, ranged from 25 days in child C5 to 94 days in child C2 (14).

Intrasample variability increased progressively over time in both the rapid- and slow-progressor infants (Fig. 5A). The pattern of synonymous and nonsynonymous substitutions was extremely varied in the first months of life, regardless of HIV-1 RNA levels, $CD4^+$-cell count, and disease progression (Fig. 5B). Indeed, from the first to the second sample, the number of intrasample synonymous substitutions decreased in children C2, C3, and C4 and increased in children C1 and C5; the number of nonsynonymous substitutions decreased in children C1 and C3 and increased in children C2, C4, and C5. The mean D_s/D_n values from the first to the second sample varied from 0 to 7.1, 6.82 to 2.86, 1.57 to 1.54, and 1.83 to 0.23 in C1, C2, C3, and C4, respectively. During this same period of time, the mean D_s/D_n values increased from 0 to 1.85 in C5, whose viral population was extremely homogeneous at days 7 and 48. Interestingly, the increase in the number of nonsynonymous substitutions was highest in C4, who was born to a mother seronegative for V3 epitopes and in whom autochthonous antibodies to V3 epitopes could be documented at the estimated age of 58 days (14). Therefore, although the changes in the V3 region during the first 6 months of life might reflect

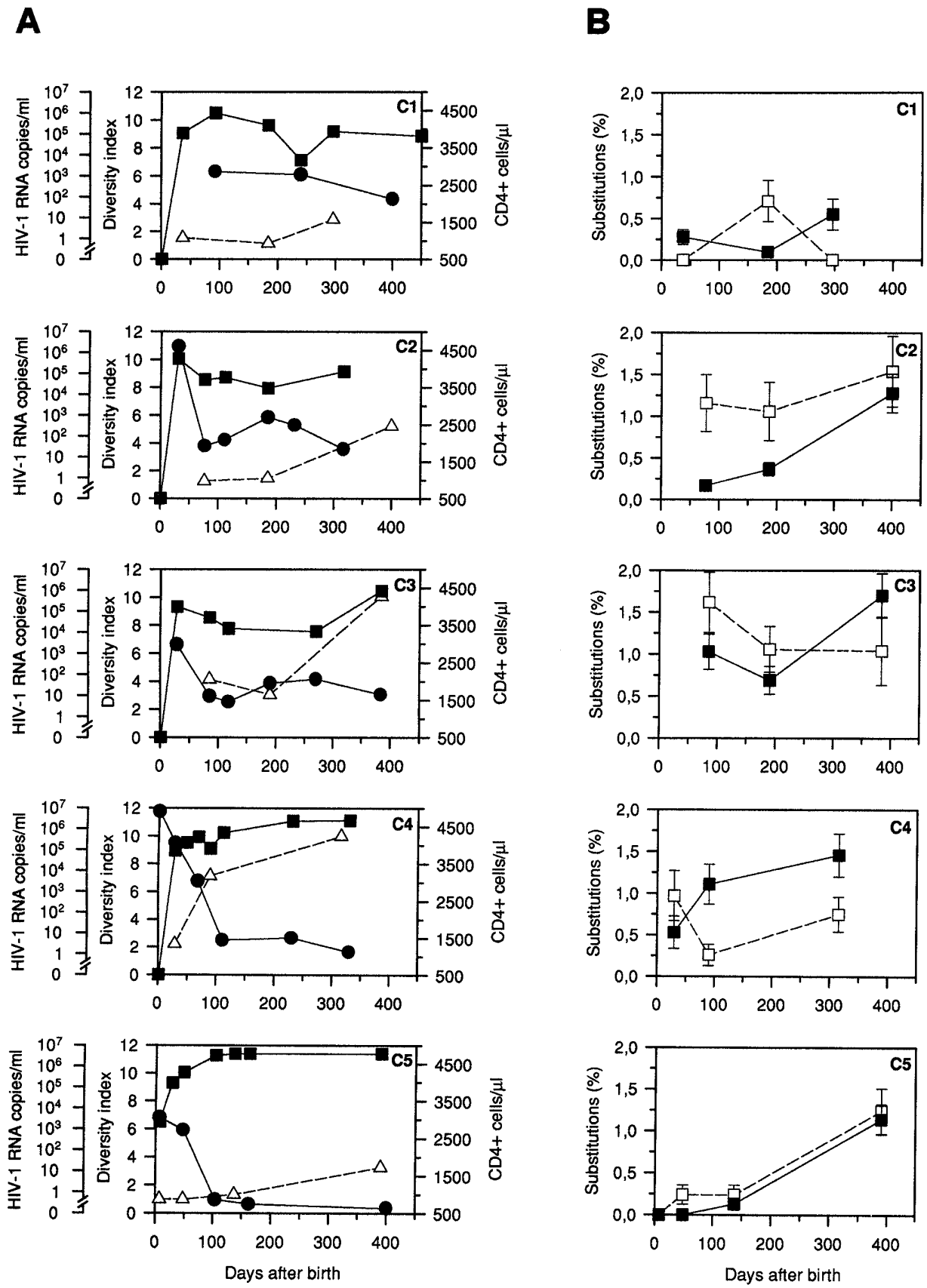

FIG. 5. Molecular and genetic parameters in three slow-progressor (C1, C2, and C3) and two rapid-progressor (C4 and C5) HIV-1 infected infants. The patient code is shown within each plot. (A) Copy number of HIV-1 RNA per milliliter of plasma (■), $CD4^+$-cell number (●), and intrasample viral diversity (△). The genetic diversity of the virus population was measured by the Simpson index (*D*), as detailed in Materials and Methods; the plot shows 1/*D* values for each sample at each time point. (B) Mean values (± standard errors) of synonymous (□) and nonsynonymous (■) substitutions. The numbers of synonymous and nonsynonymous substitutions were calculated by using the method of Nei and Gojobori (40), incorporating the Jukes-Cantor correction for multiple substitutions, and expressed as percentages.

immune selective pressures, both the genetic data and antibody detection findings strongly argue against a role for the humoral response in curtailing the virus after the initial peak in slow-progressor infants. In contrast, during the second 6 months of life, the increase in the number of nonsynonymous substitutions was comparable to that for synonymous substitutions in rapid-progressor infants C4 and C5 but exceeded that of synonymous substitutions in all three slow progressors. The mean D_s/D_n values varied from 0.23 to 0.51 in C4 and from 1.85 to 1.09 in C5 but varied from 7.1 to 0, from 2.86 to 1.20, and from 1.54 to 0.61 in C1, C2, and C3, respectively, thus suggesting a stronger positive selection for changes in slow progressors than in rapid progressors.

No relationship emerged between the evolution of the V3 domain and the viral phenotype. Indeed, analysis of the viral phenotype at the time of first positive HIV-1 detection disclosed that three infants (C1, C4, and C5) had a rapid/high-type virus according to the pattern of viral growth in primary coculture (13, 43), but only one primary isolate (from C4) displayed SI capacity in MT-2 cells. Further studies performed at the last time point of genetic analysis disclosed that no shift in the viral phenotype had occurred in slow- or rapid-progressor infants (data not shown).

DISCUSSION

Infants with perinatally transmitted HIV-1 infection show two different patterns of disease outcome within their first months of life: some develop severe symptoms of disease and rapidly progress to AIDS, while others show a variable period of clinical latency. We studied the transmission and evolution of HIV-1 in two rapid- and three slow-progressor infants by analyzing maternal samples collected at delivery and sequential samples from infants collected over their first year of life.

All but one of the infants studied were negative for viral markers at birth. This would suggest that they were most likely infected during the intrapartum period or close to the time of delivery. Only child C5 gave positive results by PCR and virus culture at first examination on day 7 after birth; in this case, transmission could have occurred either in utero or during the intrapartum period. Interpair phylogenetic analysis showed that sequences from each mother-infant pair were clearly distinct from one another and were clustered in a star-shaped phylogeny, indicating a pattern of evolution from a common ancestral sequence. Given the lack of direct epidemiological linkage between the pairs, this sequence may represent either a general ancestral subtype B virus or a particular local ancestral strain. Intrapair phylogenetic analysis supported the notion that a single V3 genetic variant initiated the infection in the infant, in agreement with observations made by others (1, 38, 54, 65). This variant constituted the predominant form within the viral population in the first sample from the child and, with the exception of one case (C5), was found to be related to a minor maternal variant in the blood, thus suggesting a selective process during transmission or the first round of viral replication in the new host. Interestingly, the predominant variants at 1 month of age in both children C1 and C4 were identical at the amino acid level to minor variants within the maternal virus population, thus suggesting that selection occurred during transmission. None of the sequences in children C2 and C3 at 3 months of age showed identity with the maternal sequences. Although transmission early in utero could not be excluded in these two cases, it appears unlikely in light of the negative findings at birth; therefore, a selection following transmission might explain these results. Reports that viral variants show different tissue distributions, and that genital secretions may harbor viral variants distinct from those in blood (44, 66), suggest the alternative explanation that transmitted variants originate from sites distinct from the peripheral blood compartment. In any case, the comparison of the sequence data with the patterns of plasma HIV-1 RNA levels (Fig. 5) suggests that viral replication in the first month of life leads to the expansion of the transmitted variant, while a selection within the host may occur only after this first phase.

The selective transmission of unglycosylated V3 variants has been demonstrated by some studies (65) although not confirmed in others (1, 54). Moreover, previous studies (43, 62) suggested that monocyte/macrophage-tropic variants are selectively transmitted from mother to child and/or selectively replicated upon transmission. In the present study, we found that V3 glycosylation sites were highly conserved in both maternal and infant sequences, except for the site upstream of the first cysteine of the V3 domain (i.e., position 14 [Fig. 4]), which was lacking in the majority of the sequences from mothers M3 and M4 and in all the sequences from the first sample of their infants. Furthermore, based on the presence of an uncharged or acidic amino acid at positions 11, 13, 25, and 29 within the V3 domain, most of the sequences in the mothers and infants were potentially of the non-SI phenotype; in agreement with this, all of the primary isolates from mothers and infants were able to grow in monocyte-derived macrophage cultures. A few maternal sequences predictive of the SI phenotype could be detected in M2, M3, and M4, whose isolates also displayed SI activity (43), but none of these sequences were found in their infants, although the isolate from C4 retained SI activity. Therefore, although no distinctive pattern could be seen between transmitted and nontransmitted variants or between rapid- and slow-progressor infants, these findings might support the notion of a positive selection of unglycosylated monocyte/macrophage-tropic variants both during transmission and during the first cycles of viral replication in the newborn.

A major difference between rapid- and slow-progressor infants is that the latter are able to curtail and modulate viral replication after the first phase of replication soon after birth (16), thus confirming the evidence that high and persistent levels of virus production are directly related to disease progression (37, 46). All of the infants in this study showed a rapid increase in plasma HIV-1 RNA content soon after birth, but this initial rise was followed by a decline in viral burden only in the three slow progressors. Both the immune response and viral phenotype may contribute in determining the different patterns of HIV-1 replication and production. Specific V3 sequences were not detected in rapid- and slow-progressor infants, suggesting that genetic polymorphism of the V3 region is not directly related to the early onset of AIDS in infants. The majority of the sequences persisted over time as the non-SI potential phenotype in both rapid- and slow-progressor infants, and accordingly, no changes in viral phenotype were documented. Only one (C4) of the two rapid progressors had an SI-type virus, and viral isolates from this child retained SI characteristics despite the absence of SI-type sequences within the V3 region. Although the presence of few variants with SI-type sequences cannot be excluded, this finding, in agreement with previous observations (9, 36), might indicate that epitopes other than V3 may contribute to the SI capability of the virus.

As the V3 domain contains recognition sites for both humoral and cellular immune responses, variations within this region would be informative about the positive selective forces exerted by the host's immune system. We found that genetic variability increased over time and did not correlate with disease progression or $CD4^+$-cell decline. However, an analysis of

the number of synonymous and nonsynonymous substitutions revealed differences between rapid and slow progressors. Indeed, during the second 6 months of life, the increase in the number of nonsynonymous substitutions was comparable to that of synonymous substitutions in the rapid-progressor infants. Conversely, during the same period of age, the increase in the number of nonsynonymous substitutions was higher than that of synonymous substitutions in all three slow-progressor infants, thus suggesting that an increase in genetic diversity in the slow, but not in the rapid, progressors correlated with positive selection for change. These findings differ from the observations of Strunnikova et al. (58) and agree with recent studies with adults showing that a greater HIV-1 population complexity and a positive selection for nonsynonymous substitutions correlates with a slower $CD4^+$-cell decline and a prolonged asymptomatic status (12, 33, 64).

Recent data support the idea that V3 genetic evolution is mainly driven by host immune constraints (33, 64). In this regard, it is noteworthy that of the two infants (C3 and C4) who lacked an N-linked glycosylation site at position 14 in their sequences soon after birth, only the slow progressor (C3) showed variants with a potential glycosylation site at this position in subsequent samples. As N-linked glycosylation sites could be important in the formation of conformational epitopes and might mask linear epitopes, thus contributing to the immune escape of viral mutant forms (2, 28, 56), the different pattern observed in the two infants might reflect different immune pressure. However, it is noteworthy that during the first 6 months of life, the patterns of synonymous and nonsynonymous substitutions varied from child to child regardless of the disease progression status; furthermore, mean D_s/D_n values were decreased in all three slow-progressor infants at the last time point, thus indicating that positive selection for changes occurred primarily in the second semester of life. This is of particular interest, because in the three slow-progressor infants viral decline occurred earlier, during the third month of life. Taking into consideration the proposal that positive selection for amino acid changes in V3 is driven mainly by host immune system forces, these findings suggest that the immune response to V3 contributes to regulating viral levels after the first semester of life but is unlikely to play a determinant role in curbing the virus after its rapid spread soon after birth. Interestingly, child C4 developed early AIDS despite the onset of antibody production against the V3 domain (14). It has been proposed that cellular immunity rather than humoral immune responses might play a role in restraining the virus after the primary infection (4, 22, 31). The finding that HIV-1-specific cytotoxic T lymphocytes are only rarely detected in infected newborns (34, 35) and our observation that positive selection for changes in V3 were detected mainly after the first semester of life suggest that factors other than the immune response to V3 play a crucial role in controlling virus replication soon after birth.

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